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(57) Abstract

The present invention relates to a peptide capable of inducing an immune response against the Zona Pellucida protein ZP3, said peptide having an amino acid sequence of 8-50 amino acid residues and comprising at least the amino acid sequence PLWLLQ or analogues thereof. More specifically, the peptides according to the invention comprise at least the amino acid sequence QPLWLLQG. Further embodiments of the invention relate to antibodies raised against the peptides according to the invention, contraceptive vaccines comprising said peptides or said antibodies, as well as a test kit for the detection of autoimmune antibodies against ZP3 in a test sample.

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NEW IMMUNOCONTRACEPTIVE PEPTIDES

The present invention relates to a peptide capable of inducing an immune response against the Zona Pellucida protein ZP3, use of said peptide to produce antibodies, monoclonal antibodies that are immunoreactive with said peptide, hybridoma cell lines producing said antibodies, use of said peptide or said antibodies for the preparation of a contraceptive vaccine, contraceptive vaccines comprising said peptides or said antibodies, use of said peptides for the detection of autoimmune antibodies against ZP3 in a sample, a diagnostic reagent comprising said antibodies and a test kit for the detection of autoimmune antibodies against ZP3 in a sample.

The Zona Pellucida (ZP) is the complex extracellular glycoprotein matrix which surrounds the mammalian oocyte. This matrix is formed during the early stages of oocyte maturation and follicular development and comprises three heavily glycosylated proteins, designated ZP1, ZP2 and ZP3. The ZP plays an important role in the fertilization process, since the first interaction between mammalian gametes is mediated by the binding of the spermatozoon to a specific receptor on the ZP. In the mouse, the ZP3 glycoprotein has been identified as the sperm receptor for complementary molecules residing in the sperm surface membrane (reviewed in Wasserman, Development 108:1-17, 1990). This ligand function of ZP3 triggers a process called the acrosome reaction resulting in the release of proteolytic enzymes which allows the spermatozoon to penetrate the zona pellucida and fertilize the oocyte through fusion with its plasma membrane.

The important role of the ZP3 glycoprotein in sperm-egg interaction in combination with its oocyte specific expression has lead to the suggestion that ZP-proteins might serve as an attractive target antigen for the development of a contraceptive vaccine (see for example Gwatkin et al. Fert. Ster. 28:871-877, 1977; Paterson and Aitken, Curr. Opinion in Immunol., 2:743-747, 1990). In many studies it has been shown that vaccination of mammalians with porcine zona pellucida proteins yield high antibodies titers against the antigens used and an accompanied infertility. Also by *in vitro* studies results have been obtained that were predictive for the contraceptive effects of ZP3-immunizations. It has been demonstrated that anti-ZP3 specific antibodies inhibit binding of male gametes to salt stored human oocytes (van Duin et al. Hum. Reprod. 9 (suppl. 4):41, 1994) and moreover also disrupt human *in vitro* fertilization (Henderson et al. Gam. Res., 1988).

Engineering a ZP3-based vaccine using natural ZP3 protein as target antigen is not possible due to the extremely limited availability of this biological material. This problem was solved by the recent cloning of the genes encoding for the ZP proteins, thus opening possibilities to generate recombinant ZP3 protein or fragments thereof. The cloning and characterization of the murine ZP3 DNA represented an important step towards this end (Ringuette et al., Proc. Natl. Acad. Sci. USA 83:4341-4345, 1986; Ringuette et al., Dev. Biol., 127:287-295, 1988). The cloning of the ZP3 genes of various mammalians and the primary amino acid sequence of the corresponding ZP3 protein have been described for human ZP3 (WO 90/15624, WO 93/14786), hamster ZP3 (Kinloch et al., Dev. Biol., 142:414-421, 1990), marmoset ZP3 (WO 94/10304), pig ZP3 (WO 93/14786;), cat ZP3 (JP-A-06014784; WO 94/11019), dog ZP3 (JP-A-

05336974; WO 94/11019), rabbit ZP3 (WO 94/11019), cow ZP3 (WO 94/11019) and cynomolgus monkey ZP3 (WO 94/11019). All thus far elucidated mammalian ZP3 amino acid sequences display a strong evolutionary conservation with amino acid homologies ranging from 65% to over 90%. The N-terminal region of these proteins are less well conserved among the different mammalian species indicating a potential role for this part of the ZP3 protein in the species specificity of the mammalian fertilization process.

A large number of mammals have been immunized with intact ZP3 proteins yielding in most cases high anti-ZP3 titers and corresponding infertility. However, reports on these studies have also mentioned the consistent observation of ovarian malfunctioning determined by a disturbed menstrual cyclicity, ovarian pathology and loss of follicles of various stages of development including the primordial follicles (see for example: Skinner et al. Endocrinology, 115:2418-2432, 1984; Upadhyay et al. Biol. Reprod. 41:665-673, 1989); Sehgal et al. Pathology 21:105-110, 1989; Dunbar et al. Fert. Ster. 52:311-318, 1989; Jones et al., J. Reprod. Fert. 95:513-525, 1992; Mahi-Brown et al. J. Reprod. Immunol. 21:29-46, 1992, Paterson et al. Biol. Reprod. 46:523-534, 1992). This malfunctioning leads to permanent sterility. Especially in humans this is a highly unwanted side effect, since the immunocontraceptive effect of a ZP3 based vaccine is only needed to control fertility, and the induced infertility should therefore be preferably reversible, either spontaneously or by manipulation. Hence, intact ZP3 is not suitable for the development of a safe contraceptive vaccine.

ZP3 displays antigenic determinants which can induce either a T cell mediated or a B cell mediated immune response. Depending on the type of response

induced, these antigenic determinants are referred to as T cell or B cell epitopes. Using the mouse as a model it has been shown that the ovarian pathology observed after vaccination with ZP proteins is a T cell mediated phenomenon. Ovarian pathology, defined as oophoritis, could be induced in mice after immunization with a small 8 amino acids T cell epitope (corresponding to amino acids 330-337 of the mouse ZP3). Transfer of the T cells of these animals to non-immunized animals also resulted in ovarian injury which strongly supports the notion that T cell epitopes account for ovarian damage after ZP3 immunizations (Rhim et al. J. Clin. Invest. 89:28-35, 1992; Luo et al. J. Clin. Invest. 92:2117-2123, 1993)

Hence, to adequately engineer a ZP3 based contraceptive vaccine, there is a strong need for those fragments of the ZP3 protein, which do not contain the pathogenic T cell epitopes of ZP3. It is an object of the invention to provide for a peptide which corresponds to a single antigenic determinant of the ZP3 protein, whereby said antigenic determinant does not induce a pathogenic T cell mediated immune response.

The present invention provides for such a peptide. The peptide according to the invention has an amino acid sequence of 8-50 amino acid residues and comprises at least the amino acid sequence PLWLLQ (SEQ ID NO:1) or analogues thereof.

More specifically the peptide according to the invention comprise the amino acid sequence QPLWLLQG (SEQ ID NO:2) or analogues thereof.

The flanking sites of the amino acid sequence PLWLLQ (SEQ ID NO:1), more specifically QPLWLLQG (SEQ ID NO:2), in the peptide according to the invention may correspond to the native flanking sites of amino acid 24-29, more particularly 23-30, of the amino acid

sequence of human ZP3 or may correspond to non-native flanking sites made up of any random amino acid sequence.

The peptides according to the invention have an amino acid sequence of 8-50, preferably 8-35, more preferably 8-25 amino acid residues. Much more preferred are peptides having an amino acid sequence of 8-15 amino acid residues. Particularly preferred are peptides having an amino acid sequence of 8 or 12 amino acid residues.

Multimeres of the peptide according to the invention such as for example a dimere or a trimere are also within the scope of the invention. Such multimeres provide a multitude of the specific amino acid sequences PLWLLQ and/or QPLWLLQG to which an immune response can be elicited.

As used herein, analogues are those peptides which contain substitutions or replacements, insertions or deletions in the amino acid sequences PLWLLQ or QPLWLLQG (SEQ ID NO:1 and 2) or polymeric forms of said sequences, with the proviso that these analogues are immunoreactive with the monoclonal antibodies produced by the hybridoma cell line deposited with the European Collection of Animal Cell Cultures (herein further referred to as ECACC), Port Down, Salisbury (UK) on March 24, 1994 under deposit number 94032402. The substitutions or replacements do not necessarily have to constitute conservative substitutions as described by M.O. Dayhoff (Atlas of protein structure , vol. 5, suppl. 3, Natl. Biomedical Research Foundation, 1978), but can also constitute non-conservative amino acid substitutions which result in a mimitope of the amino acid sequences PLWLLQ or QPLWLLQG (SEQ ID NO:1 or 2).

A mimitope as used herein is an amino acid sequence, that differs from the sequences given in SEQ ID NO:1 and 2, but which is capable of inducing other

antibodies that recognize the same epitope as is recognized by the monoclonal antibodies produced by hybridoma cell line deposited with the ECACC under deposit number 94032402. The procedure to identify such mimitopes has been described by Geysen et al. (Proc. Natl. Acad. Sci. USA 81:3998-4002, 1984; J. Immunol. Methods 134:23-33, 1987). This technology is well known in the art and kits with reagents and materials are commercially available.

For example, peptides having an amino acid sequence of 8-50 amino acid residues and which comprise at least the amino acid sequence PLWFWQ (SEQ ID NO:3) or PMWTLQ (SEQ ID NO:4) are analogues that are immunoreactive with the antibodies produced by the hybridoma cell line deposited with the ECACC under deposit number 94032402.

Suitable petides according to the invention are peptides having the amino acid sequence QPLWLLQG (SEQ ID NO:2), PQPLWLLQ (SEQ ID NO:5), PLWLLQGG (SEQ ID NO:6), LCYPQPLWLLQGGASHPETS (SEQ ID NO:7) or ADGAPMWTLQGAAGA (SEQ ID NO:8) or mixtures thereof.

A preferred peptide for the development of a contraceptive vaccine is a peptide having the amino acid sequence QPLWLLQG (SEQ ID NO:2).

Following the generation of high antibody titers after vaccination with a peptide according to the invention, disturbance of ovarian function or other indications for ovarian injuries have not been observed. The peptides according to the invention do not elicit a pathogenic T cell mediated immune response and are therefore particularly suitable for the development of a contraceptive vaccine for immunization against ZP3.

A peptide which does not elicit a pathogenic T cell mediated immune response against ZP3 has been described by Millar et al. (Science 246:935-938, 1989). Millar identified a small antigenic determinant, corresponding

to amino acid 336-342 of the mouse ZP3 amino acid sequence, which after active immunization of female mice induced a contraceptive effect without inducing ovarian pathology. This peptide, however, is totally different from the peptides according to the invention. The peptides according to the invention comprise the amino acid sequence given in SEQ ID NO:1 or 2, or analogues thereof, which corresponds to amino acid 24-29, in particular amino acid 24-30 of the human ZP3 amino acid sequence. Nowhere does Millar suggest or hint to a peptide according to the invention, or to the fact that such a peptide will not elicit a pathogenic T cell mediated immune response.

The peptides according to the invention are very suitable for development of a contraceptive vaccine for active immunization against ZP3. A contraceptive vaccine for the active immunization against ZP3 comprises an effective amount of one or more peptides according to the invention and a pharmaceutical acceptable carrier. Administration of the contraceptive vaccine to a female mammalian, especially a human female, will induce an immune response that is directed to the corresponding antigenic determinant on the ZP3 protein of the oocytes and not to all the other antigenic determinants present on the ZP3. Hence, a more controlled immune response can be obtained with a contraceptive vaccine comprising the peptides according to the invention, as compared to a vaccine based on intact ZP3. The resulting antibodies inhibit the binding of sperm cells to the oocyte, thus inducing infertility.

Furthermore, the peptides according to the invention can be used to develop a contraceptive vaccine for passive immunization against ZP3. A contraceptive vaccine for the passive immunization against ZP3 is based on an effective amount of one or more antibodies

which have been raised against a peptide according to the invention, and a pharmaceutical acceptable carrier.

In particular antibodies raised against a peptide according to the invention, which are immunoreactive with amino acid sequence of SEQ ID NO:1 and/or 2, are very suitable for use in a contraceptive vaccine for passive immunization against ZP3.

Preferably the antibodies raised against a peptide according to the invention are monoclonal antibodies.

A preferred antibody is the monoclonal antibody produced by the hybridoma cell line deposited with the ECACC under deposit number 9432402. More preferred monoclonal antibodies are human or humanized monoclonal antibodies to develop a contraceptive vaccine for passive immunization against ZP3.

Antibodies raised against a peptide according to the invention as well as cell lines producing such antibodies also fall within the scope of the invention.

Administration of such a contraceptive vaccine to a female mammalian, especially a human female, provides the female with a homogenous population of antibodies which are immunoreactive with the amino acid sequence given in SEQ ID NO:1 and/or 2 as well as the corresponding antigenic determinant displayed by amino acid 23-30, more in particular amino acid 24-29 of the amino acid sequence of ZP3, but which antibodies do not react with the other antigenic determinants present on the ZP3. The binding of the antibodies to the respective antigenic determinant on the ZP3 of the oocytes inhibits sperm cells from binding to said oocyte, thus inducing infertility.

In another embodiment of the invention the peptides according to the invention are suitable for use in a test kit to detect the presence of autoimmune antibodies directed to amino acid 24-29, more particular 23-30, of the amino acid sequence of ZP3. To detect these

autoimmune antibodies, a serum sample is taken from the subject and contacted with one or more peptides according to the invention, and optionally a diagnostic reagent comprising antibodies according to the invention, preferably monoclonal antibodies according to the invention. If autoimmune antibodies are present, they will react with said peptides. The reaction that can take place can be, amongst others, an agglutination reaction, a competition reaction or an inhibition reaction. Detection of the reaction can be accomplished by labelling either the peptide or the antibody according to the invention, depending on the type of reaction that takes place, with a suitable detecting agent.

To carry out, for instance, an inhibition reaction for the detection of the afore-mentioned autoimmune antibodies in a test sample, the test kit to be used comprises one or more peptides according to the invention coated on a solid support, and a diagnostic reagent comprising a labelled monoclonal antibody according to the invention or fragment thereof. The binding of this reagent to the peptide on a solid support can be competed by the autoimmune antibodies in the test sample.

Supports which can be used are, for example, the inner wall of a microtest well or a cuvette, a tube or capillary, a membrane, filter, test strip or the surface of a particle such as, for example, a latex particle, an erythrocyte, a dye sol, a metal sol or metal compound as sol particle, a carrier protein such as Bovine Serum Albumin (BSA) or Keyhole Limpet Hemocyanine (KLH).

Detection agents which can be used to label the peptide according to the invention or the reagent antibody of the diagnostic reagent are, inter alia, a radioactive isotope, a fluorescent compound, an enzyme, a dye sol, metal sol or metal compound or other sol as sol particle.

Test kits comprising one or more peptides according to the invention and optionally a diagnostic reagent comprising antibodies, preferably monoclonal antibodies, according to the invention can be used to diagnose unwanted infertility resulting from autoimmune antibodies directed to ZP3.

Furthermore, these test kits can be used to monitor the antibody levels in female mammalians who are treated with a vaccine according to the invention.

The preparation of the peptides according to the invention can be effected by means of one of the known organic chemical methods for peptide synthesis or with the aid of recombinant DNA techniques.

The organic chemical methods for peptide synthesis are considered to include the coupling of the required amino acids by means of a condensation reaction, either in homogeneous phase or with the aid of a so-called solid phase.

The condensation reaction can be carried out as follows:

a) condensation of a compound (amino acid, peptide) with a free carboxyl group and protected other reactive groups with a compound (amino acid, peptide) with a free amino group and protected other reactive groups, in the presence of a condensation agent;

b) condensation of a compound (amino acid, peptide) with an activated carboxyl group and free or protected other reaction groups with a compound (amino acid, peptide) with a free amino group and free or protected other reactive groups.

Activation of the carboxyl group can take place, inter alia, by converting the carboxyl group to an acid

halide, azide, anhydride, imidazolide or an activated ester, such as the N-hydroxy-succinimide, N-hydroxy-benzotriazole or p-nitrophenyl ester.

The most common methods for the above condensation reactions are: the carbodiimide method, the azide method, the mixed anhydride method and the method using activated esters, such as described in The Peptides, Analysis, Synthesis, Biology Vol. 1-3 (Ed. Gross, E. and Meienhofer, J.) 1979, 1980, 1981 (Academic Press, Inc.).

Preparation of peptides according to the invention using the "solid phase" is for instance described in J. Amer. Chem. Soc. 85:2149 (1963) and Int. J. Peptide Protein Res. 35:160-214 (1990). The coupling of the amino acids of the peptide to be prepared usually starts from the carboxyl end side. For this method a solid phase is needed on which there are reactive groups or on which such groups can be introduced. This can be, for example, a copolymer of benzene and divinylbenzene with reactive chloromethyl groups, or a polymeric solid phase rendered reactive with hydroxymethyl or amine-function.

A particularly suitable solid phase is, for example, the p-alkoxybenzyl alcohol resin (4-hydroxy-methyl-phenoxy-methyl-copolystrene-1% divinylbenzene resin), described by Wang (1974) J. Am. Chem. Soc. 95:1328. After synthesis the peptides can be split from this solid phase under mild conditions.

After synthesis of the desired amino acid sequence, detaching of the peptide from the resin follows, for example, with trifluoromethanesulphonic acid or with methanesulphonic acid dissolved in trifluoroacetic acid. The peptide can also be removed from the carrier by transesterification with a lower alcohol, preferably methanol or ethanol, in which case a lower alkyl ester of the peptide is formed directly. Likewise, splitting

with the aid of ammonia gives the amide of a peptide according to the invention.

The reactive groups which may not participate in the condensation reaction are, as stated, effectively protected by groups which can be removed again very easily by hydrolysis with the aid of acid, base or reduction. Thus, a carboxyl group can be effectively protected by, for example, esterification with methanol, ethanol, tertiary butanol, benzyl alcohol or p-nitrobenzyl alcohol and amines linked to solid support.

Groups which can effectively protect an amino group are the ethoxycarbonyl, benzyloxycarbonyl, t-butoxy-carbonyl (t-boc) or p-methoxy-benzyloxycarbonyl group, or an acid group derived from a sulphonic acid, such as the benzene-sulphonyl or p-toluene-sulphonyl group, but other groups can also be used, such as substituted or unsubstituted aryl or aralkyl groups, for example benzyl and triphenylmethyl, or groups such as ortho-nitrophenyl-sulphenyl and 2-benzoyl-1-methyl-vinyl. A particularly suitable _-amino-protective group is, for example, the base-sensitive 9-fluorenyl-methoxycarbonyl (Fmoc) group (Carpino & Han, J. Amer. Chem. Soc. 92:5748, 1970).

A more extensive account of possible protecting groups can be found in The Peptides, Analysis, Synthesis, Biology, Vol. 1-9 (Eds. Gross, Udenfriend and Meienhofer) 1979 - 1987 (Academic Press, Inc.).

It is necessary also to protect the _-amino group of lysine and it is advisable to protect the guanidine group of arginine. Customary protective groups in this connection are a Boc-group for lysine and a Pmc- or Pms- or Mbs-group or Mtr-group for arginine.

The protective groups can be split off by various conventional methods, depending on the nature of the particular group, for example with the aid of trifluoroacetic acid or by mild reduction, for example with hydrogen and a catalyst, such as palladium, or with HBr in glacial acetic acid.

As already indicated above, the peptides according to the invention can likewise be prepared with the aid of standard recombinant DNA techniques. For this purpose, a nucleic acid sequence which codes for a peptide according to the invention or a multimere of said peptide is inserted into an expression vector. Suitable expression vectors are, amongst others, plasmids, cosmids, viruses and YAC's (Yeast Artificial Chromosomes) which comprise the necessary control regions for replication and expression. The expression vector can be brought to expression in a host cell. Suitable host cells are, for instance, bacteria, yeast cells and mammalian cells. Such techniques are well known in the art (Sambrooke et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989).

Antibodies according to the invention can be prepared according to standard techniques. Procedures for immunizing animals, e.g. mice with peptides and selection of hybridomas producing immunogen specific monoclonal antibodies are well known in the art (see for example Coligan et al. (eds), Current protocols in Immunology, 1992; Kohler and Milstein, Nature 256:495-497, 1975; Steenbakkers et al., Mol. Biol. Rep. 19:125-134, 1994). In short, a selected animal is given multiple injections with peptides conjugated to an immunogenic carrier protein, like for example keyhole limpet hemocyanine (KLH). Immune responses of the immunized animal can be readily assessed in serum using

microtitre plates coated with antigen of interest whereas hybridomas are generated by electrofusion of myeloma cells with mouse B cells and subsequent selection in medium containing the appropriate selective agents for this purpose.

Methods for production of humanized monoclonal antibodies may involve genetic engineering technologies which can be adopted to reshape or humanize the antibodies. For example, the complementarity determining regions (CDR's) comprising the antigen binding site of murine monoclonal antibodies are inserted into human antibody framework regions, thereby generating human antibodies in which the CDR-regions are derived from the original murine antibody. This method of 'CDR-grafting' has been used successfully for therapeutic purposes in a large number of instances like, for example, antibodies against the interleukin 2 receptor (Queen et al., Proc. Natl Acad. Sci. USA 86:10029-10033, 1989), epidermal growth factor receptor (Kettleborough et al., Prot. Engin. 4:773-783, 1991), Carter et al., Proc. Natl Acad. Sci. USA 89:4285-4289, 1989), carcinoembryonic antigen (Bosslet et al., Br. J. Cancer 65:234-238, 1992) and various viruses (Tempest et al., BioTechnology 9:266-271, 1991; Co et al., Proc. Natl Acad. Sci. USA 88:2869-2873, 1991; Maeda et al., Human antibodies and hybridomas 2:124-134, 1991.

A much preferred method for generating monoclonal antibodies according to the invention is the immunization of transgenic animals which have been manipulated to express complete human antibodies. Examples for this technology have been described by Green et al. (Nature Genetics 7:13-21, 1994) and Lonberg et al. (Nature 368:856-859, 1994). By immunizing these types of transgenic animals with the peptides according to the invention, and using standard hybridoma-technology as outlined above, human monoclonal antibodies can be generated. Such human antibodies are

highly preferred for the passive immunization of human females.

Contraceptive vaccines according to the invention comprise an effective immunogenic amount of the aforementioned peptides or antibodies and a pharmaceutical acceptable carrier. The term "effective immunogenic amount" as used herein is defined as the amount sufficient to induce an immune response with a contraceptive effect in the female mammalian. The amount of peptide or antibody will depend on the route of administration, the time of administration, the species of the female as well as age, general health and diet.

In general, a dosage of 0.01 to 1000 µg peptide per kg body weight, preferably 0.5 to 500, more preferably 0.1 to 100 µg peptide can be used.

Pharmaceutical acceptable carriers are well known to those skilled in the art and include, for example, sterile saline, lactose, sucrose, calcium phosphate, gelatin, dextrin, agar, pectin, peanut oil, olive oil, sesame oil and water.

Optionally, the vaccine according to the invention may comprise one or more adjuvants. These adjuvants are used as a non-specific irritant to attract or enhance an immune response. Suitable adjuvants include, amongst others, aluminium hydroxide, aluminium phosphate, amphigen, tocophenols, monophosphenyl lipid A, muramyl dipeptide and saponins such as Quill A. The amount of adjuvant added depends on the nature of the adjuvant itself.

In addition, to enhance the immunogenecity of the peptide according to the invention, said peptide can be coupled to an immunogenic carrier protein, such as Tetanus Toxoid (TT) and KLH. The coupling of the peptide can be accomplished either via reactive groups present in the peptide itself, or by introducing such a reactive

group, for example an extra cysteine residue, at the C-terminus of the peptide.

The term "immunogenic carrier protein" as used herein is defined as proteins that are very immunogenic and as such trigger the immune system of the host, thus increasing the immunogenic effect of the peptides according to the invention. The immunogenic carrier proteins as such are functionally different from, and should not be confused with, the afore-mentioned pharmaceutical acceptable carriers.

Furthermore the vaccine according to the invention may also comprise one or more stabilizers such as for example carbohydrates including sorbitol, mannitol, starch, sucrose, dextrin, and glucose, proteins such as albumin or casein, and buffers like alkaline phosphates.

Any female mammalian may be treated with the contraceptive vaccine according to the invention. Preferably human females are treated with the vaccine according to the invention. The administration protocol can be optimized in accordance with standard vaccination practice. Suitable administration routes are intramuscular injections, subcutaneous injections, intravenous injections or intraperitoneal injections, oral administration and nasal sprays.

Legends to the figures

Figure 1.
ELISA experiment showing the binding, expressed as the optical density at 450 nm, of monoclonal antibody ZP4A to different peptides. The peptides have the amino acid sequences given in SEQ ID NO:2, 7 and 9-11 (numbers 1 to 5 in the figure).

Figure 2.
ELISA experiment showing that binding of monoclonal antibody ZP4A to microtiter plates coated with recombinant ZP3 is inhibited by peptide QPLWLLQG (SEQ ID No. 2), indicated by closed circle, and not by a control peptide, indicated by triangle. The experiment was carried out in duplo and the average results are shown. The binding is expressed as the optical density at 450 nm.

Figure 3.
Immunofluorescent staining of human zonae pellucidae with monoclonal antibodies. The immunostaining is expressed as the exposure time determined by a photomultiplier. Antibodies used: 1 = NP11-1A (control IgG_1); 2 = ZP4A. The experiment was carried out in triplicate. Mean values ± standard deviations are shown.

Figure 4.
Inhibition of binding of human spermatozoa to human zonae pellucidae by monoclonal antibodies. Antibodies tested: 1 = NP11-1A (control IgG_1); 2 = ZP4A. The average number of spermatozoa bound to 4 human oocytes ± standard deviations are given.

Figure 5.
Results of vaccination of marmoset monkeys with the peptide QPLWLLQG (SEQ ID NO:2). The upper panel shows

the antibody titres against the immunized peptide (indicated by open circle) and human recombinant ZP3 (indicated by closec circle) after the primary (P) and first booster (B) immunizations. The bottom panel shows an example of an animal with normal ovarian function as evidenced by the cyclicity of plasma progesterone levels, determined by radio-immunoassay prior to and after the start of the vaccinations.

Example 1

Synthetic peptides were produced by solid phase synthesis using procedures described by Fields and Noble (Int. J. Pept. Prot. Res. 35:160-214, 1990) and were coupled to bovine serum albumin (BSA) using glutaraldehyde. Briefly, 100 µl peptide (1 mg/ml) was mixed with 25 µl BSA solution (12 mg/ml) after which 25 µl 60 mM glutaraldehyde was added. Following incubation at room temperature for 1 hour 15 µl 0.5 M glycine pH 8.0 was added and after 30 minutes at room temperature the conjugated protein fraction was purified by chromatography (PD-10 columns, Pharmacia). The conjugated peptides were further diluted to a concentration of 1 µg/ml in 0.05 M Sodium carbonate/bicarbonate) buffer (coating buffer), pH 9.6; 100 µl/well and coated overnight at room temperature to polystyrene microtitre plates. Two peptides having the amino acid sequence given in SEQ ID NO:2 and 7 as well as three control peptides having the amino acid sequences given in SEQ ID NO:9-11 were thus synthesized. Immunodetection of the peptides was done using 1 µg/ml monoclonal antibody produced by the hybridoma cell line deposited with the ECACC under deposit number 94032402 (herein further referred to as mAb ZP4A) in PBST (phosphate buffered saline + 0.05% Tween 20, pH 7.5) for 1 hour. After three wash steps with PBST the plates were incubated with 100 µl/well goat-anti-mouse IgG-horse radish peroxidase conjugate diluted 1 in 5000 in PBST for 1 hour. After a single wash step with PBST and two times washing with water 100µl/well tetramethylbenzidine (TMB) substrate buffer was added and incubated for 15-30 minutes at room temperature. The staining reaction was terminated by adding 100 µl 4N sulphuric acid (H_2SO_4). The absorbance of the wells was determined at 450 nm.

The results presented in Figure 1 demonstrate that mAb ZP4A specifically recognises the peptides according to the invention (peptide number 1 and 2, respectively),

whereas no binding is observed with the control peptides (number 3 to 5, respectively).

Example 2

Polystyrene microtitre plates were coated with serum free cell supernatant of Chinese hamster ovary cells producing recombinant human ZP3 (van Duin et al., Biol. Reprod. *in press*, 1994). Towards this end, this medium was diluted 1:10 with coating buffer (see example 1) and plates were incubated overnight at room temperature (100 µl well). After washing three times with PBST, the plates were co-incubated with mAb ZP4A (1 µg/ml) and different concentrations of the peptide QPLWLLQG (SEQ ID NO:2) and a control peptide.

Immunodetection of the bound monoclonal antibody ZP4A was by incubation of the plates with 100 µl/well goat-anti-mouse IgG-horse radish peroxidase (HRP) conjugate diluted 1 in 5000 in PBST for 1 hour. After a single wash step with PBST and two times washing with water 100 µl/well TMB substrate buffer was added and incubated for 15-30 minutes at room temperature. The staining reaction was terminated by adding 100 µl 4N sulphuric acid (H_2SO_4). The absorbance of the duplicate wells was determined at 450 nm.

The results are presented in Figure 2 and demonstrate that the peptide according to the invention competitively inhibits the binding of mAb 4A to human recombinant ZP3, whereas this binding is not inhibited by a control peptide. It can be concluded that the epitope recognized by mAb ZP4A is present on both the recombinant ZP3 as well as the peptide according to the invention, indicating that antibodies raised against a peptide according to the invention are able to react with the corresponding antigenic determinant present on ZP3.

Example 3

Human oocytes were obtained from *in vitro* fertilisation programs. Briefly, the oocytes that had failed to fertilise, as determined by the absence of cleavage were stored in oocyte storage solution (OSS, 20 mM HEPES, 1.5 M MgCl2.6H2O, 0.1% dextran, 10% glycerol, 0.1% polyvinylpyrrolidone, pH 7.2-7.4) and stored at 4 °C until further use. Before use in human egg-fluorescence assays (hEFA's) the oocytes, *i.e.* zonae pellucidae, are rinsed by transfer through three droplets of 250 µl PBS-EFA (PBS, 0.5% bovine serum albumin (BSA), 1 % polyvinylpyrrolidone-40, 100 U/ml penicillin., 100 µl/ml streptomycin) using a micropipette. To investigate whether mAb ZP4A specifically recognises ZP3 residing in the matrix with the ZP1 and ZP2 molecules of the human zona pellucida, oocytes were incubated in droplets of 25-30 µl PBS-EFA with 100 µg/ml antibody (sample 2, Figure 3) for 1 hour at 37 °C in humidified box. To determine the level of aspecific binding oocytes were also incubated under identical conditions with monoclonal antibody NP11-A1 (sample 1, Figure 3), an irrelevant antibody of similar isotype. Three wash steps in 250 µl PBS-EFA with 5-10 minutes intervals each were carried out prior to incubation in 25-30 µl PBS-EFA plus conjugated rabbit anti-mouse immunoglubulins conjugated to FITC (fluorescein isothiocyanate) (1:100 dilution, incubation for 1 hour at 37 °C in humidified box). Following again three wash steps in droplets of 250 µl with intervals of 5-10 minutes the oocytes are introduced in 250 µl anti-fading solution (Johnson, J. Immunol. Meth. 43:349-350, 1981). To avoid fading effects during the final analysis each oocyte is subsequently mounted on a single object slide, surrounded by a small ring of vaseline and enclosed by coverslip. Each oocyte was analysed by fluorescent microscopy. Immediately following exposure of the oocytes to fluorescent light a photomultiplier was used

to record the exposure time required to make a photograph. Using this procedure, the exposure time calculated by the photomultiplier is a measure for the amount of antibody bound to the oocyte. Long exposure times indicate no or limited antibody binding whereas specific binding to the oocyte results in short exposure times.

The results presented in Figure 3 show that incubation of human oocytes, *i.e.* zonae pellucidae, with mAb ZP4A yields a relatively high level of fluorescence whereas virtually no fluorescent staining is found with the control monoclonal antibody NP11-1A of identical isotype. It can be concluded from these results that the epitope for mAb ZP4A is present in native human ZP3 protein.

Example 4

Human salt-stored oocytes were rinsed as described in example 3 followed by three wash steps in droplets of human tubal fluid medium plus 0.3% human serum albumin (HTF-medium; see Quinn et al., Fertil. Steril. 44:493-498, 1985). For each analysis 4-5 human oocytes were used that were co-incubated for 1 hour in a single droplet of 25 µl HTF-medium containing the test compounds, *i.e.* antibodies, to be analysed (sample 1: isotype control monoclonal antibody NP11-1A; sample 2: mAb ZP4A, both 250 µg/ml). The incubation was carried out at 37°C in a humidified box in the presence of 5% CO_2. After washing the oocytes three times in droplets 250 µl HTF-medium they were collected in a volume of 25 µl and mixed with 25 µl HTF medium containing $2x10^6$ Percoll purified motile human spermatozoa/ml that had been capacitated at 37°C at least for three hours. The 50 µl incubation droplet was covered with paraffin oil and incubated for 4 hours at 37 °C in a 5% CO_2-incubator.

Bound and free sperm cells were separated by a dextran-gradient centrifugation step. The pellet + 100 µl supernatant was mixed with 1 ml fixation and staining solution [0.9 ml BWW-medium (in: Daniel, J.C.jr.(ed), Methods of mammalian embryology, W.H. Freeman, SAn Fransisco, pp. 86-116, 1971), 0.1 ml 10% glutaraldehyde, 1-10 µl 2 mg/ml bismenzimid H33258 (Hoechst)] and incubated overnight at 4°C. Finally, the oocytes were collected from this solution using microscope and micropipette, and incubated in anti-fading solution and mounted on object slides as described in example 3. The number of bound spermatozoa was determined by fluorescent microscopy and counting of the average number of cells per mm^2 in two focused plains on top and bottom of the fixed oocytes.
The results of this experiment are given in Figure 4. In contrast to the control monoclonal antibody, mAb ZP4A inhibits binding of human spermatozoa to human oocytes. Thus the antibodies raised agaist the peptides according to the invention react with native zonae pellucidae on human oocytes and inhibit the binding of the spermatozoa to the zonae pellucidae, thereby inducing infertility.

Example 5

Immunizations of female marmoset monkeys (*Callithrix jacchus*) with peptide QPLWLLQG (SEQ ID NO:2). First a cysteine residue was coupled to the C-terminal site of the peptide to provide a reactive site in the peptide. Via this reactive site the peptide was coupled to the carrier protein tetanus toxoid (TT) using sulpho-SMCC (N-succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate). First, accessible -SH groups were formed on the peptide by adding 6 mg 2-mercaptoethanolamine to 4 mg peptide dissolved in 5 ml 0.1 M phosphate buffer pH 6.0, 5 mM EDTA. After incubation for 1 hour at 37 °C , the peptide was purified by Sephadex G-25 chromatography and stored at 4°C.

Carrier protein was activated by dissolving 4 mg in 5 ml 50mM sodium borate buffer, pH 7.0 followed by adding 2 mg sulpho-SMCC and incubation for 1 hour at 30°C. Sephadex G-25 chromatography was adopted to purify the protein. Fractions containing the activated carrier protein were pooled together and concentrated to 5 ml using an Amicon B-15 microconcentrator. To allow conjugation the fractions containing the peptide were added to the activated concentrated TT and incubated on a rocker for 20 h at 4 °C. The conjugated material was subsequently purified using a Sephacryl S-400 column equilibrated with 0.1 M Tris HCl, 1 mM $MgCl_2$ pH 7.0 and a flow rate of 20 ml/h.

Immunization: three months prior to immunization marmosets are monitored for menstrual cyclicity and normal ovarian function by measuring serum progesterone levels bi-weekly. As a primary immunization 200 µg conjugate in PBS emulsified with non-ulcerative Freund's adjuvant containing 250 µg N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) was given s.c. at several sites in a total volume of 0.2 ml. Booster injections are prepared in a similar manner except that MDP is omitted.
To monitor both immune response and ovarian function immunized animals were bled twice a week to determine anti-peptide QPLWLLQG and anti-recombinant ZP3 antibodies as well as serum progesterone levels, respectively.
The anti-peptide response was measured using maleic anhydrate activated plates (Pierce). Plates were coated overnight at room temperature (RT) with 5 mg peptide/ml PBS pH 8.0. (100 µl/well). After flicking out the peptide solution the wells were washed twice with 200 µl blocking buffer (3% BSA, 0.05% Tween 20 in PBS) and incubated with a further 200 µl blocking buffer for 1 h at RT. After this third wash step two-fold dilution's of the sample to be tested were added ranging from a 1/10

dilution to 1/20480 (75 µl, 2 h at RT). After three wash steps with wash buffer (0.1% BSA, 0.05% Tween 20 in PBS)) 100 µl of an anti-monkey HRP conjugate (diluted 1/1000) was added to each well followed by incubation at RT for 1h. Further development of the plates was as described in Example 1.

ELISAs for recognition of human recombinant ZP3 were essentially as outlined in Example 1.

Marmoset plasma progesterone levels were determined by use of a non-extraction radio-immunoassay. Each assay was performed in triplicate with 2.5 µl plasma diluted to 150 µl with 0.1 M Phosphate citrate buffer, pH 6.0, containing 0.1% gelatine (PCB). Sheep anti-progesterone antibody, 100 µl of 1/10000 dilution in PCB, and 100 µl iodinated progesterone tracer were added and the solution was incubated for 3 h at 25 °C. A second antibody, 100 µl Of 1/64 dilution in PCB donkey anti-sheep serum (Scottish Antibody Production Unit (SAPU), Scotland), was then added, followed by 100 µl of 1/3200 dilution of normal sheep serum (SAPU), and the solution was incubated overnight at 4 °C. Finally, 1 ml 4% polyethylene glycol, 0.2% Triton X-100 in 0.9% saline was added and centrifuged at 1500 g for 30 min at 4 °C. After the supernatant was discarded, the tubes were counted in a multigamma counter (LKB).

The results presented in Figure 5 demonstrate that immunization with a peptide according to the invention can induce an immune response. The resulting antibodies recognize the intact human ZP3 protein as well as the petide QPLWLLQG (upper panel), whereas the cyclicity of the plasma progesterone levels indicate a normal ovarian function.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: AKZO NOBEL N.V.
(B) STREET: Velperweg 76
(C) CITY: Arnhem
(E) COUNTRY: The Netherlands
(F) POSTAL CODE (ZIP): 6824 BM
(G) TELEPHONE: 04120-66376
(H) TELEFAX: 04120-50592
(I) TELEX: 37503 akpha nl

(ii) TITLE OF INVENTION: New contraceptive peptides

(iii) NUMBER OF SEQUENCES: 11

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Pro Leu Trp Leu Leu Gln
1 5

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Gln Pro Leu Trp Leu Leu Gln Gly
1 5

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Pro Leu Trp Phe Trp Gln
1 5

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Pro Met Trp Thr Leu Gln
1 5

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Pro Gln Pro Leu Trp Leu Leu Gln
1 5

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Pro Leu Trp Leu Leu Gln Gly Gly
1 5

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Leu Cys Tyr Pro Gln Pro Leu Trp Leu Leu Gln Gly Gly
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Ala Ser His Pro Glu Thr Ser
15 20

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Ala Asp Gly Ala Pro Met Trp Thr Leu Gln Gly Ala Ala
1 5 10

Gly Ala
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(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Lys Val Thr Leu Ala Glu Gln Asp Pro Asn Glu Leu Asn
1 5 10

Lys Ala Cys Ser Phe Ser Lys
15 20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Thr Asp Asp Ala Leu Val Tyr Ser Thr Phe Leu Leu His
1 5 10

Asp Pro Arg Pro Val Gly Asn Leu
15 20

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Pro Ser Asp Thr Ser Val Val Leu Leu Gly Val Gly Leu
1 5 10

Ala Val Val Val Ser
15

0

Claims

1. Peptide having an amino acid sequence of 8-50 amino acid residues, said peptide comprising at least the amino acid sequence PLWLLQ (SEQ ID No. 1) or analogues thereof, provided that said analogues are immunoreactive with monoclonal antibodies produced by the hybridoma cell line deposited with the ECACC under deposit No. 94032402.

2. Peptide according to claim 1, characterised in that said peptide comprises at least the amino acid sequence QPLWLLQG (SEQ ID No. 2) or analogues thereof.

3. Peptide according to claim 1 or 2, characterised in that said peptide has an amino acid sequence QPLWLLQG (SEQ ID No. 2).

4. Peptide according to claim 1 or 2, characterised in that said peptide has an amino acid sequence PQPLWLLQ (SEQ ID No. 5), PLWLLQGG (SEQ ID No. 6) or LCYPQPLWLLQGGASHPETS (SEQ ID No. 7).

5. Peptide according to claim 1 or 2, characterised in that said peptide has an amino acid sequence ADGAPMWTLQGAAGA (SEQ ID No. 8).

6. Use of a peptide according to any of the claims 1-5 for producing antibodies.

7. Antibodies raised against a peptide according to any of the claims 1-5.

8. Antibody according to claim 7, characterised in that said antibody has a specific binding capacity

for an epitope comprising the amino acid sequence PLWLLQ or QPLWLLQG.

9. Antibody according to claim7 or 8, characterized in that said antibody is monoclonal.

10. Antibody according to claim 9, characterized in that said antibody is produced by the hybridoma cell line deposited with the ECACC under deposit No. 94032402.

11. Cell lines producing antibodies according to claims 7-10.

12. Hybridoma cell line deposited with the ECACC under deposit No. 94032402.

13. Peptides according to any of the claims 1-5 and/or antibodies according to any of the claims 7-10 for use as a therapeutical substance.

14. Pharmaceutical composition comprising one or more peptides according to any of the claims 1-5 or one or more antibodies according to claims 7-10, and a pharmaceutical acceptable carrier.

15. Contraceptive vaccine comprising one or more peptides according to any of the claims 1-7 or one or more antibodies according to any of the claims 7-10, and a pharmaceutical acceptable carrier.

16. Method for detecting autoimmune antibodies in the serum of a patient comprising the steps of:
a) collecting a serum sample from the patient,
b) contacting said sample to one or more peptides according to any of the claims 1-5 and optionally a diagnostic reagent comprising one or more

antibodies according to the invention,
c) determination of the presence of said autoimmune antibodies by a suitable detection reaction.

17. A peptide according to any of the claims 1-5 or an antibody according to claims 7-10 for use as a diagnostic substance.

18. A test kit for the detection of autoimmune antibodies in a test sample, said kit comprising one or more peptides according to any of the claims 1-7 and optionally a diagnostic reagent comprising monoclonal antibodies according to claims 7-10.

19. A diagnostic reagent comprising monoclonal antibodies according to any of the claims 7-10 labelled with a detection agent.

Figure 1

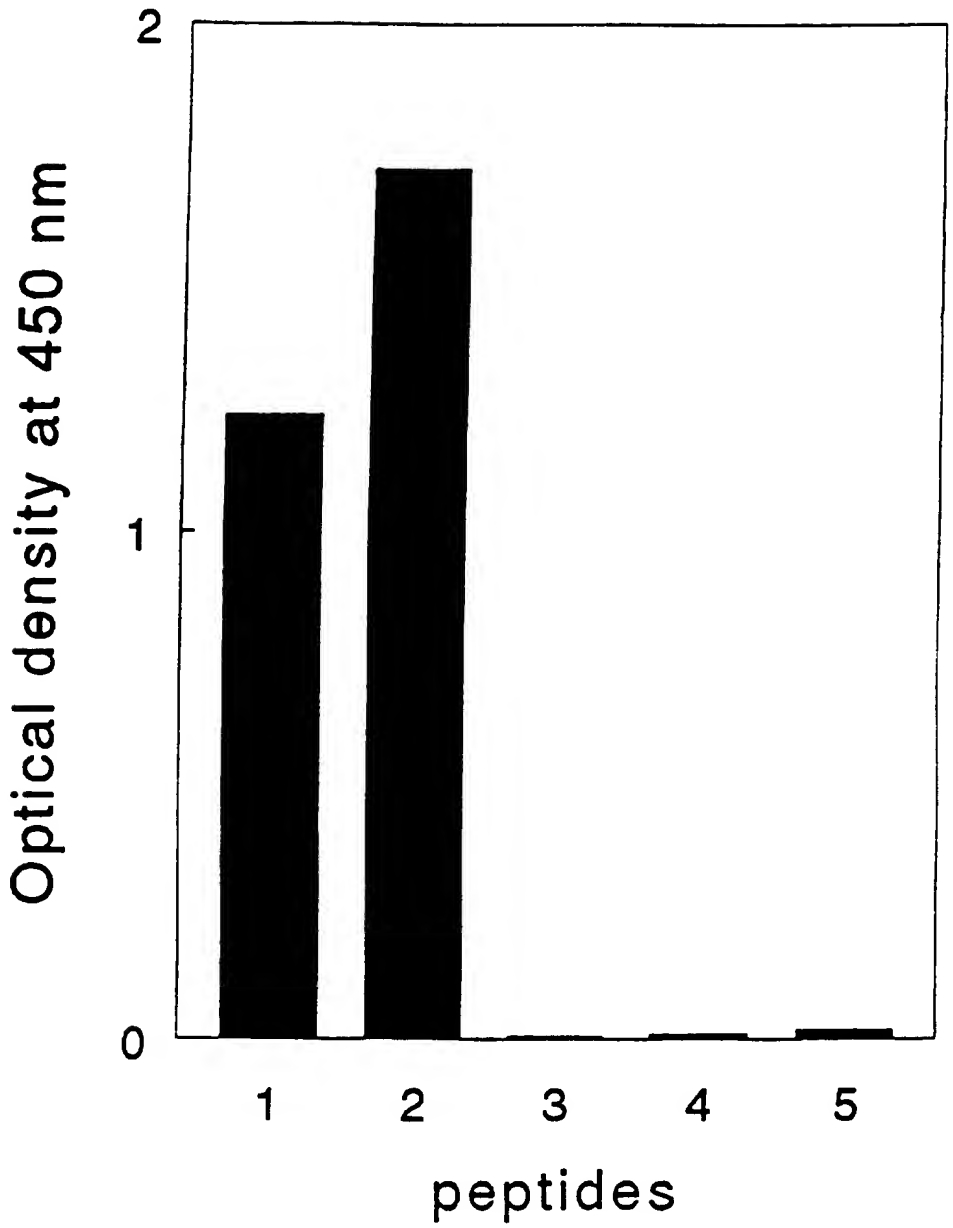
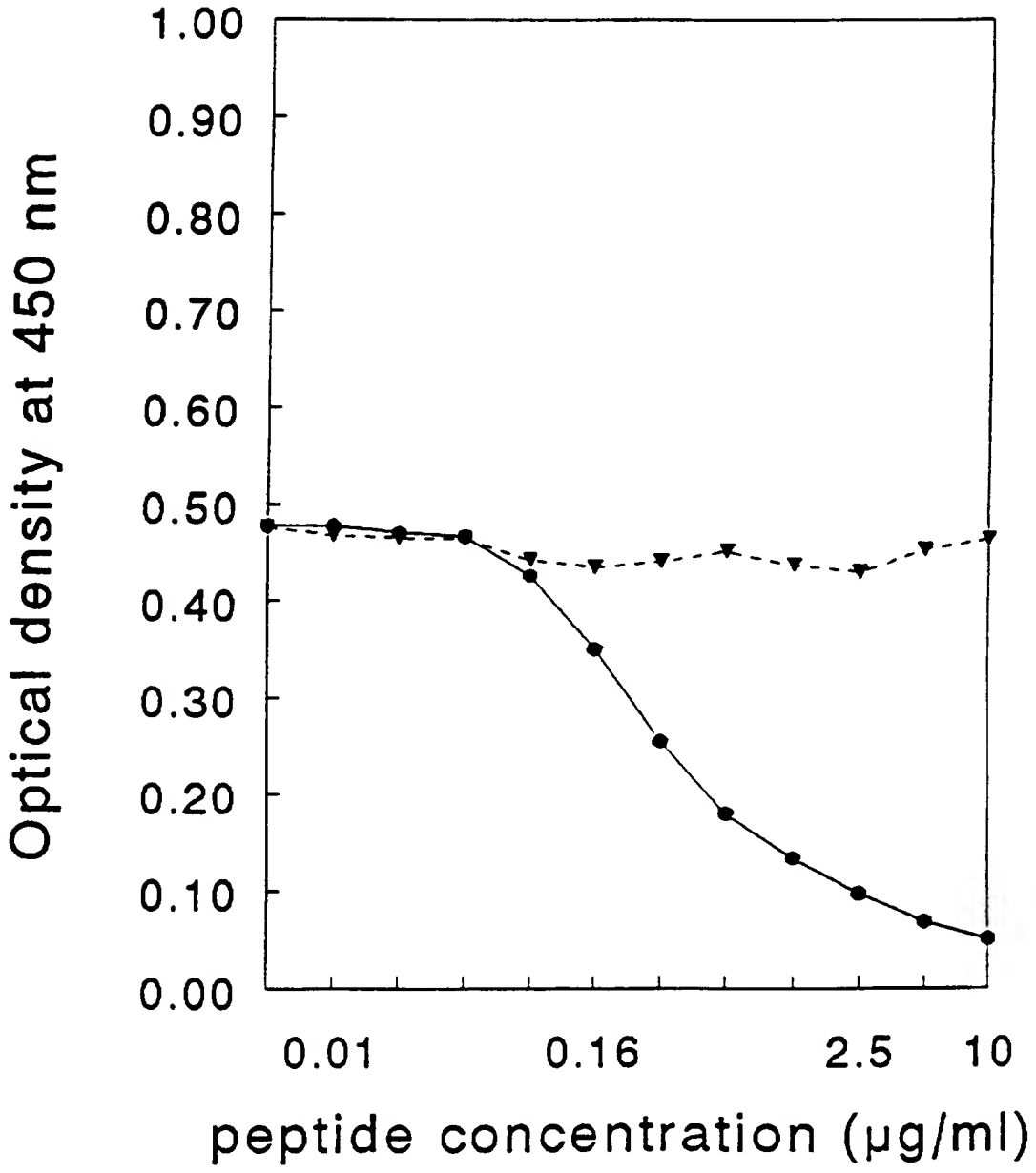


Figure 2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0.60
0.50
0.40
0.30
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0.10
0.00
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0.16
2.5
10
peptide concentration (µg/ml)

Figure 3

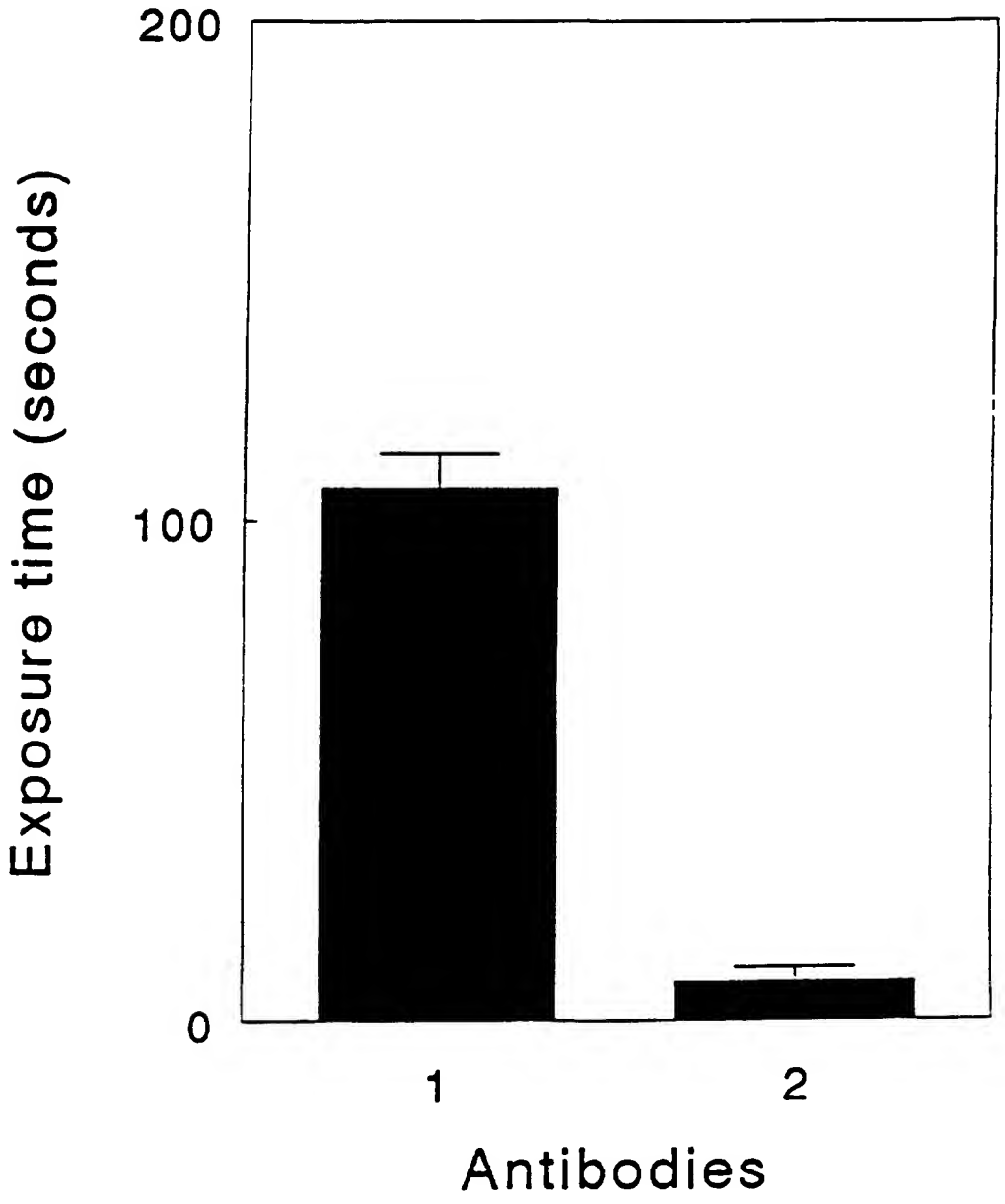


Figure 4

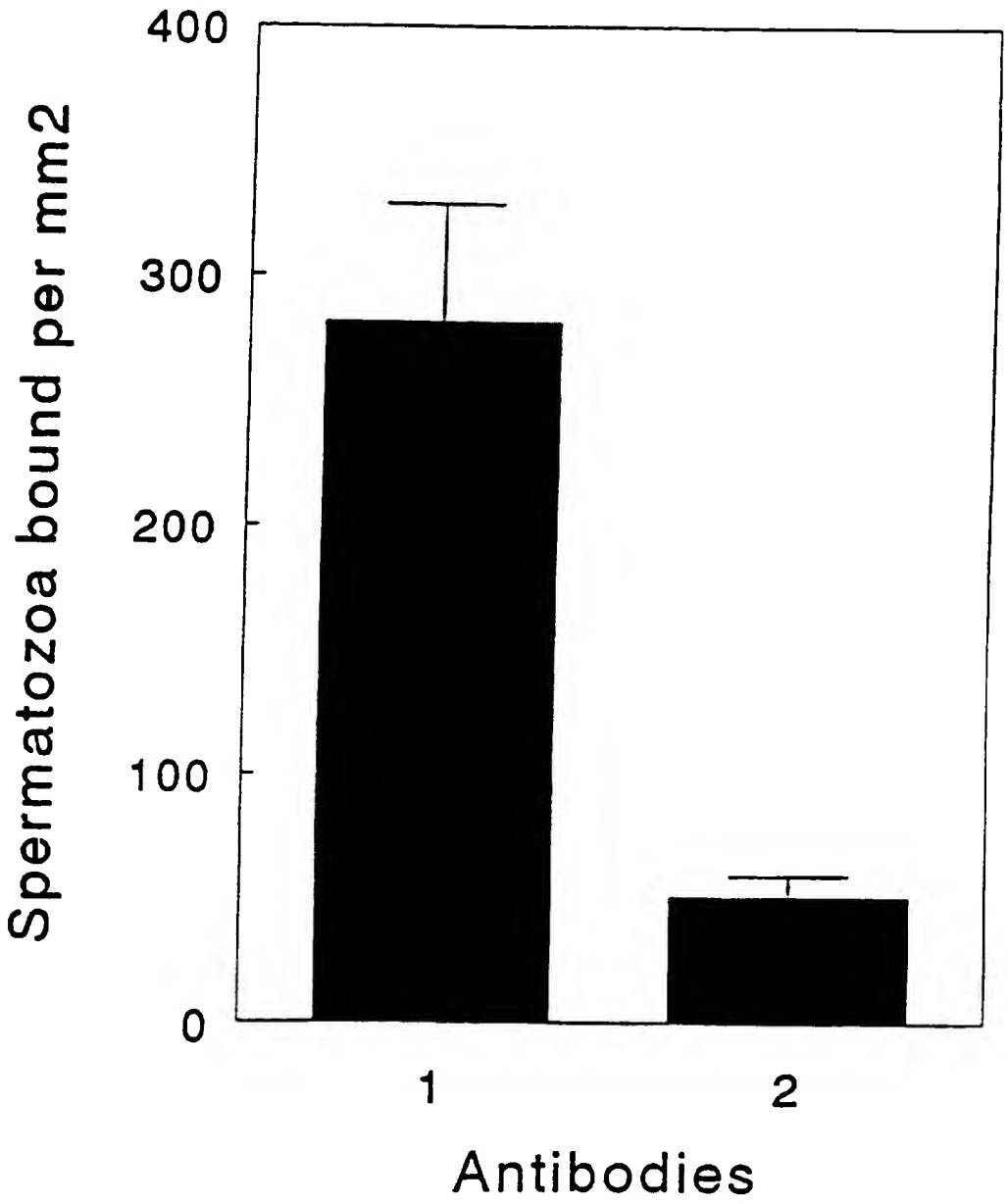
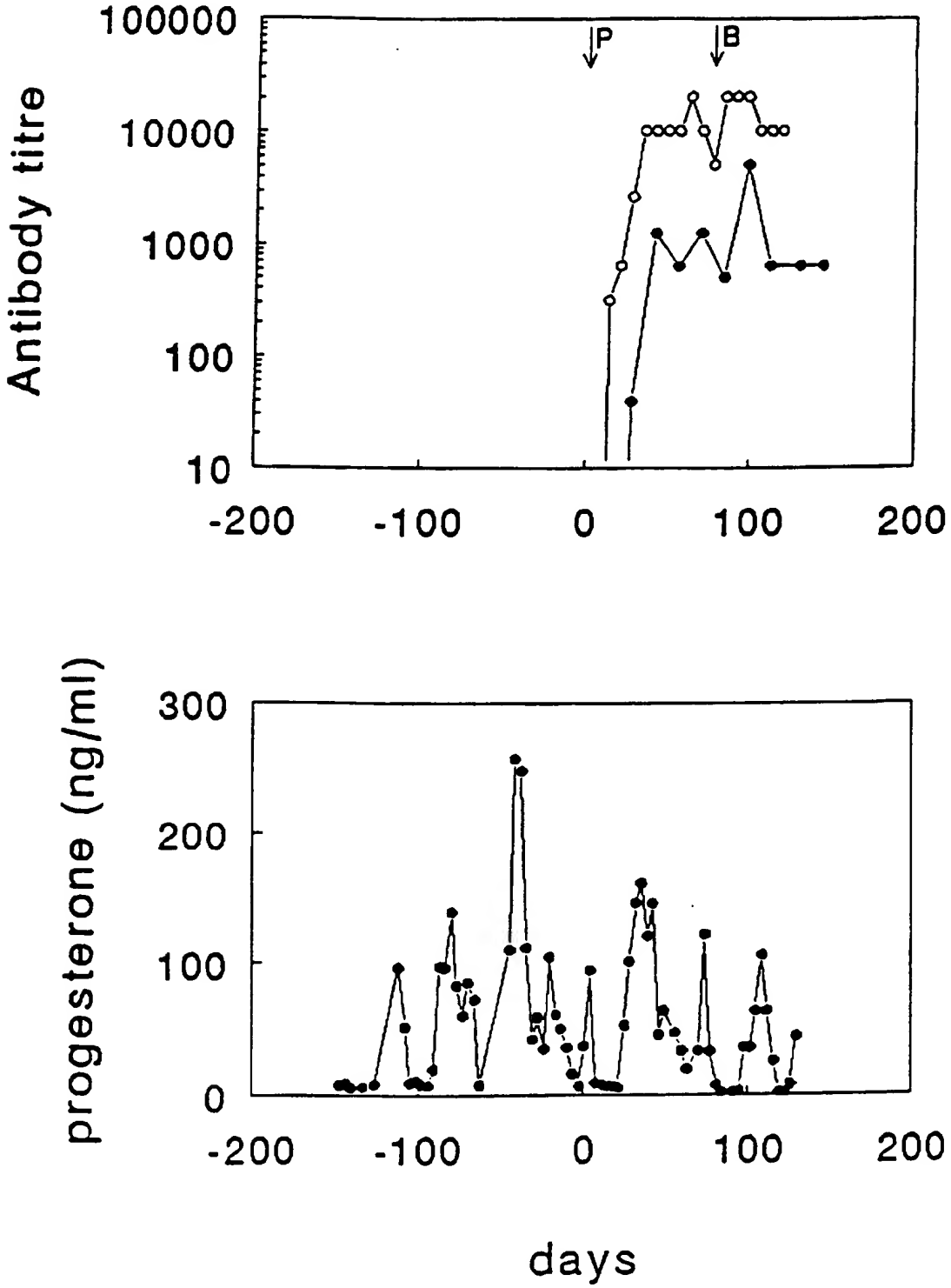


Figure 5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10
Antibody titre
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-200
-100
0
100
200
300
200
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0
progesterone (ng/ml)
-200
-100
0
100
200
days

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/EP 95/03311

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/705 C07K16/28 C12N5/12 G01N33/577 A61K38/08
A61K38/04 A61K38/16 A61K39/395 A61K39/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,92 03548 (AKZO N.V.) 5 March 1992 see page 1, line 1 - page 5, line 2 ---	1-8, 13-19
X	DATABASE WPI Section Ch, Week 9438 Derwent Publications Ltd., London, GB; Class B04, AN 94-305384 & JP,A,06 217 777 (TONEN CORP) , 9 August 1994 see abstract --- -/--	1-3, 13-15

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search
19 December 1995

Date of mailing of the international search report
05. 01. 96

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INTERNATIONAL SEARCH REPORT

Inter mal Application No
PCT/EP 95/03311

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Week 9430 Derwent Publications Ltd., London, GB; Class B04, AN 94-245693 & JP,A,06 179 698 (TONEN CORP) , 28 June 1994 see abstract	1-3, 13-15
X	DATABASE WPI Section Ch, Week 9408 Derwent Publications Ltd., London, GB; Class B04, AN 94-061479 & JP,A,06 014 784 (TONEN CORP) , 25 January 1994 cited in the application see abstract	1-3, 13-15
X	DATABASE WPI Section Ch, Week 9407 Derwent Publications Ltd., London, GB; Class B04, AN 94-051146 & JP,A,05 336 974 (TONEN CORP) , 21 December 1993 cited in the application see abstract	1-8, 13-15
X	DATABASE WPI Section Ch, Week 9333 Derwent Publications Ltd., London, GB; Class B04, AN 93-260855 & JP,A,05 176 771 (TONEN CORP) , 20 July 1993 see abstract	1-3, 13-15
Y	WO,A,89 03399 (ZONAGEN, INC.) 20 April 1989 see page 15, line 5 - page 20, line 17	1-15
Y	WO,A,90 15624 (THE UNITED STATES OF AMERICA) 27 December 1990 cited in the application see page 27, line 6 - page 36, line 16	1-15

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/EP 95/03311

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9203548	05-03-92	AU-B-	8328591	17-03-92
		CA-A-	2090486	28-02-92
		CN-A-	1060499	22-04-92
		HU-A-	64394	28-12-93
		JP-T-	6500690	27-01-94
		NZ-A-	239518	25-03-94
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		AU-B-	630862	12-11-92
		AU-B-	2536088	02-05-89
		EP-A-	0396552	14-11-90
		EP-A-	0599822	01-06-94
		JP-T-	3502571	13-06-91
WO-A-9015624	27-12-90	AU-B-	636895	13-05-93
		AU-B-	5826790	08-01-91
		CA-A-	2058999	13-12-90
		EP-A-	0477226	01-04-92
		JP-T-	5500654	12-02-93